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Antioxidant Activity of Caffeic Acid through a Novel Mechanism under UVA Irradiation

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Summary **Effect of caffeic acid on the formation of hydroxyl radicals was examined during xanthone-mediated photosensitization. The reaction was performed on irradiation (λ = 365 nm) of the standard reaction mixture containing 15 μM xanthone, 0.1 M 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) and 20 mM phosphate buffer (pH 7.4) using electron paramagnetic resonance (EPR) with spin trapping. Caffeic acid inhibited the formation of hydroxyl radicals. Caffeic acid hardly scavenged both hydroxyl radicals and superoxide radicals under conditions employed in this paper in spite of its ability to act as a hydrogen donor or a reagent for the aromatic hydroxylation, because high concentration of DMPO trapped hydroxyl radicals overwhelmingly. Furthermore, caffeic acid inhibited the formation of hydroxyl radicals in the standard reaction mixture with EDTA under UVA irradiation. Accordingly, the inhibitory effect of caffeic acid on the formation of hydroxyl radicals in the standard reaction mixture under UVA irradiation is not due to its ability to chelate iron. Thus, the inhibitory effect of caffeic acid seems to occur in the standard reaction mixture under UVA irradiation through a novel antioxidation activity, *i.e.*, ability to quench the exited xanthone.**

Key Words**: radicals, xanthone, quenching, UVA, caffeic acid**

Introduction

Of the various oxidative stresses, UV irradiation is one of the primary factors. Chronic exposure to solar UV irradiation of mammalian skin induces a number of biological responses, including erythema, edema, sunburn cell formation, hyperplastic responses, photoaging and skin cancer development [*1–3*]. Increasing evidences showed that free radicals may be involved in acute sunburn reactions [*4–6*]. Indeed, hydrogen peroxide, $^1O_2$, $O_2^{-\bullet}$ and nitric oxide were observed under UV irradiation [*7*, *8*]. Since UVB (315–280 nm) radiation constitutes only 5% of the solar UV radiation that reaches the surface of the earth, skin damages are not caused entirely by the UVB. Sufficient evidences indicated that UVA (400–315 nm) radiation, which accounts for the major portion of the solar UV radiation also leads to skin damages. The UVA-induced damages would be mediated by endogenous and/or exogenous photosensitizers.

Photosensitizers, xanthone and its derivatives have been extensively examined for the UVA-induced DNA damage [*9*, *10*]. It has been also known that xanthone mediates the photosensitized decomposition of fatty ester hydroperoxides to produce oxyl radicals [*11*] and hydroxyl radicals [*12*]. We chose xanthone-mediated photosensitization as a model system to investigate the skin damages by UVA.

Several papers have shown that polyphenols scavenge free radicals through hydrogen-donation and aromatic hydroxylation [*13–24*]. On the other hand, other studies have indicated that polyphenols inhibit the formation of free radicals and the propagation of free radical reactions through the chelation of transition-metal ions [*23–29*]. The purpose of the work described here is to examine antioxidation activities of polyphenols through the other mechanism, *i.e.*,

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quenching an exited photosensitizer.

Chlorogenic acid is an ester of caffeic acid with quinic acid (Fig. 1). It is found naturally in various agricultural products such as coffee beans, potatoes and apples. Ferulic acid (Fig. 1) occurs in rice, wheat, olives, citrus fruits and leaves and many other plants. Thus, it is of interest to examine the influence of the polyphenols on the formation of reactive oxygens by UVA in view of their widespread occurrence in food products.

Materials and Methods

Chemicals

Xanthone and disodium ethylenediaminetetraacetate (EDTA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO), caffeic acid, chlorogenic acid, D-(−)-quinic acid, ferulic acid, salicylic acid methyl ester and vanillic acid were from Tokyo Kasei Kogyo Co. LTD (Tokyo, Japan). Benzoic acid was obtained from Nakarai Chemicals, LTD (Kyoto, Japan). Salicylic acid was from Katayama Chemical (Osaka, Japan). Superoxide dismutase (SOD) and catalase were from Sigma-Aldrich Co. (St. Louise, Mo.).

EPR measurements

The electron paramagnetic resonance (EPR) experiments were carried out on a JES-FR 30 Free Radical Monitor (JEOL Ltd., Tokyo, Japan). Operating conditions of the EPR spectrometer were: power, 4 mW; modulation width, 0.1 mT; time constant, 0.3 s. Magnetic fields were calculated by the splitting of MnO (ΔH_{3-4} = 8.69 mT).

The standard reaction mixture

The standard reaction mixture contained 15 μM xanthone, 0.1 M DMPO and 20 mM phosphate buffer (pH 7.4) in a quartz test tube (100 mm long × 8 mm i.d.). The DMPO was used as a spin trapping reagent. The 0.1 M DMPO hardly absorbed light at 365 nm. The standard reaction mixtures were exposed to 13 J/cm^2 UVA light under aerobic conditions using a 400 W UV lamp and a LXO365 bandpass filter (365 nm) (ASAHI SPECTRA Co., Tokyo, Japan). After 10 min irradiation, the aqueous samples were aspirated into a Teflon tube centered in an EPR microwave cavity. And then, EPR spectra were measured.

The Fenton reaction mixture

The Fenton reaction mixture contained 1 mM H_2O_2, 1 mM $FeSO_4$ $(NH_4)_2SO_4$, 0.1 M DMPO and 20 mM phosphate buffer (pH 7.4). The reaction was started by adding $FeSO_4$ $(NH_4)_2SO_4$. After 2 min reaction, the aqueous samples were aspirated into a Teflon tube centered in an EPR microwave cavity. And then, EPR spectra were measured.

Fluorescence spectroscopy

The fluorescence spectra of the samples were taken using a 650-10S fluorescence spectrophotometer (Hitachi, Ltd., Tokyo, Japan). The sample contained 15 μM xanthone (or 15 μM xanthone with 10 μM caffeic acid) in 50 mM phos-

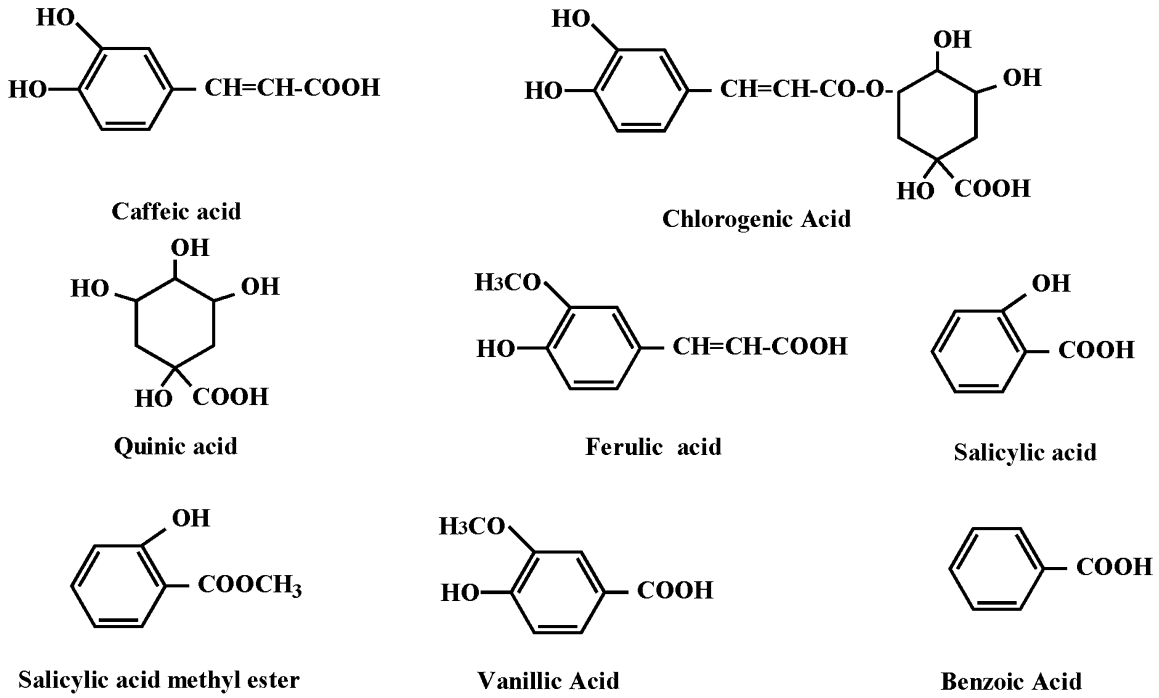


Fig. 1. Chemical structures of caffeic acid and its related compounds.

phate buffer (pH 7.4). The excitation wavelengths used were 320 nm, 340 nm and 365 nm.

Results

Effect of caffeic acid on the formation of hydroxyl radicals in the standard reaction mixture under UVA irradiation

A previous our paper showed that DMPO traps hydroxyl radicals formed in the standard reaction mixture under UVA (365 nm) irradiation [*12*]. Several papers indicated that DMPO/OH radical adduct forms in the reaction of singlet oxygen with DMPO [*30–34*]. To examine whether or not the DMPO/OH radical adduct observed in the standard reaction mixture under UVA (365 nm) irradiation forms through the reaction of singlet oxygen with DMPO, catalase (or SOD) was added. On addition of SOD, the EPR peak height of the DMPO/OH radical adduct was enhanced in the standard reaction mixture under UVA (365 nm) irradiation [*12*]. While, addition of catalase resulted in the decrease of the EPR peak height of the DMPO/OH radical adduct in the standard reaction mixture under UVA (365 nm) irradiation [*12*]. Accordingly, the DMPO/OH radical adduct observed in the standard reaction mixture under UVA (365 nm) irradiation seems to be derived from hydrogen peroxide and superoxide anions.

To study effects of caffeic acid on the formation of hydroxyl radicals, EPR measurements were performed for the standard reaction mixture with caffeic acid (or without caffeic acid) under UVA (365 nm) irradiation. On addition of 1 mM caffeic acid, the EPR peak height of the DMPO/OH radical adduct decreased to 6 ± 2% of the standard reaction mixture under UVA irradiation.

Effect of caffeic acid on the formation of hydroxyl radicals in the standard reaction mixture (or the Fenton reaction mixture) with EDTA

To know whether caffeic acid inhibits the formation of hydroxyl radicals through chelation of iron ions or not, EPR measurements were performed for the standard reaction mixture (or the Fenton reaction mixture) with EDTA (or without EDTA). On addition of caffeic acid (10 μM), the formation of hydroxyl radicals was inhibited in the standard reaction mixture with EDTA (0.1 mM) under UVA irradiation (Fig. 2B). The EPR peak heights of the DMPO/OH radical adduct decreased with increase of caffeic acid concentration in the standard reaction mixture with EDTA (or without EDTA) (Fig. 3). Fifty percent-inhibition concentration (IC_{50}) of caffeic acid was 8.8 μM in the standard

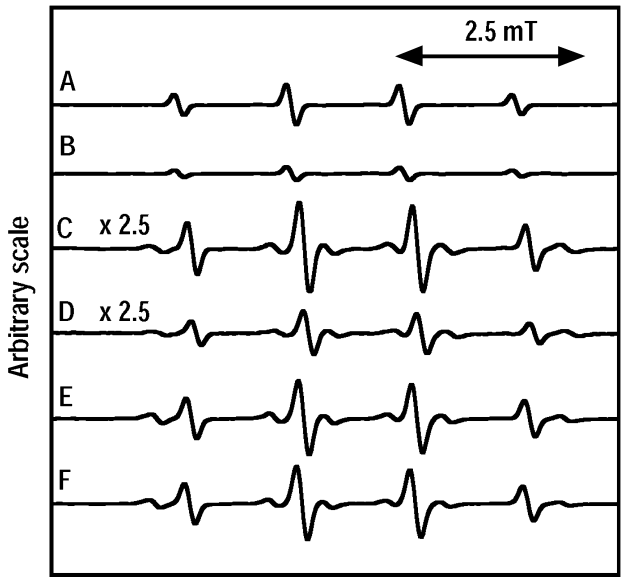


Fig. 2. Effect of caffeic acid on the formation of hydroxyl radicals. Reaction and EPR conditions were as described in **Materials and Methods**. (A) standard reaction mixture with 0.1 mM EDTA; (B) standard reaction mixture with 10 μM caffeic acid and 0.1 mM EDTA; (C) Fenton reaction mixture at ×2.5 scale; (D) Fenton reaction mixture with 1 mM caffeic acid at ×2.5 scale; (E) Fenton reaction mixture with 1 mM EDTA; (F) Fenton reaction mixture with 1 mM caffeic acid and 1 mM EDTA.

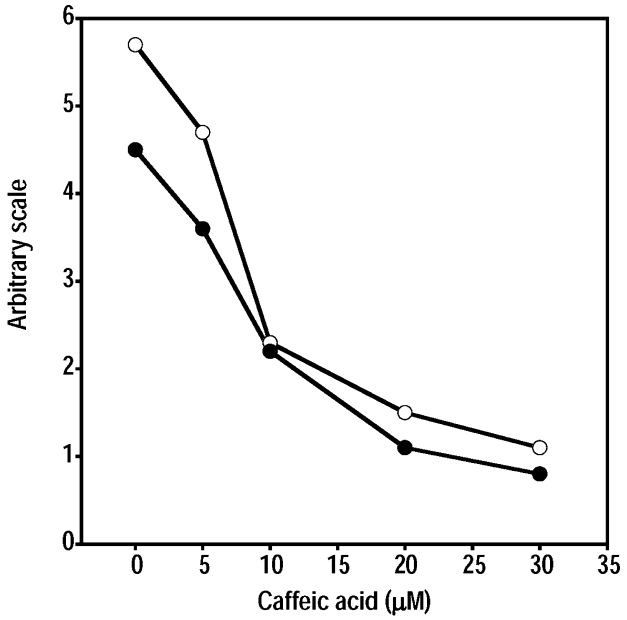


Fig. 3. Caffeic acid concentration dependence of the formation of hydroxyl radicals in the standard reaction mixture with EDTA (or without EDTA) under UVA irradiation. Reaction and EPR conditions were as described under **Materials and Methods**. (closed circle) standard reaction mixture without EDTA; (open circle) standard reaction mixture with EDTA (0.1 mM).

Table 1. Fifty percent-inhibition concentration (IC_{50}) and absorbance (365 nm) at IC50 for caffeic acid related compounds.

Compound	IC_{50} (μM)	Absorbance at IC_{50}
Caffeic Acid	8.8	0.006
Chlorogenic Acid	1000.0	2.327
Ferulic Acid	1100.0	0.401
Salicylic Acid	308.0	0.0006
Salicylic Acid Methyl Ester	1100.0	0.002
Vanillic Acid	320.0	0.0054

reaction mixture without EDTA (Table 1).

On the other hand, on addition of caffeic acid (1 mM), the formation of hydroxyl radicals was inhibited in the Fenton reaction mixture (43 ± 7% of the Fenton reaction mixture) (Fig. 2D). The inhibitory effect of caffeic acid was reduced in the Fenton reaction mixture with EDTA (95 ± 3% of the Fenton reaction mixture with EDTA) (Fig. 2F).

Effect of caffeic acid on the formation of hydroxyl radicals in the standard reaction mixture with SOD under UVA irradiation

To know whether or not caffeic acid scavenges the $O_2^{-\bullet}$, EPR measurements were performed for the standard reaction mixture with SOD under UVA irradiation. On addition of caffeic acid (1 mM) to the standard reaction mixture with SOD under UVA irradiation, the EPR peak height of the DMPO/OH radical adduct decreased to 13 ± 7% of the standard reaction mixture with SOD under UVA irradiation.

Absorbances of caffeic acid at 365 nm

To assess the effects of the UVA absorption of caffeic acid on the inhibition, absorbances at 365 nm were measured for caffeic acid at IC_{50} (Table 1). Caffeic acid hardly absorbed the light at 365 nm.

Effect of caffeic acid related compounds on the formation of hydroxyl radicals in the standard reaction mixture under UVA irradiation

Effect of caffeic acid related compounds on the formation of hydroxyl radicals was examined in the standard reaction mixture under UVA irradiation. On addition of chlorogenic acid [or ferulic acid, or salicylic acid, or salicylic acid methyl ester, or vanillic acid], the EPR peak height of the DMPO/OH radical adduct decreased to 41 ± 3% [or 52 ± 2%, or 19 ± 1%, or 49 ± 6%, or 18 ± 2%] of the standard reaction mixture under UVA irradiation (Fig. 4). On the other hand, quinic acid (or benzoic acid) showed no effect (Fig. 4).

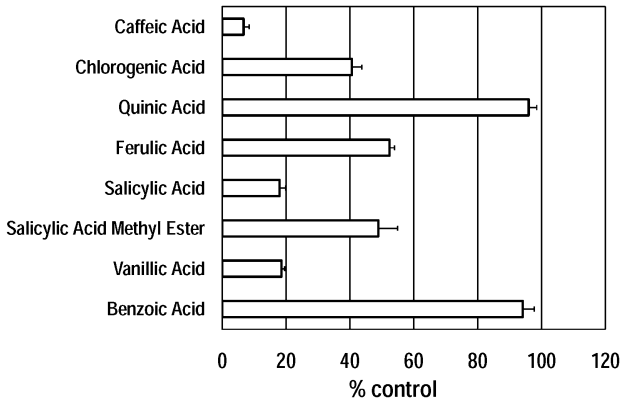


Fig. 4. Effect of caffeic acid and its related compounds on the formation of hydroxyl radicals in the standard reaction mixture under UVA irradiation. Caffeic acid, (1 mM) and its related compounds (1 mM) were added to the standard reaction mixture. The control value 100% represents the level of DMPO/OH radical adduct formed in the standard reaction mixture under UVA irradiation. The respective values are means ± SD of three determinations. Reaction and EPR conditions were as described under **Materials and Methods**.

Fifty percent-inhibition concentrations (IC_{50}) of the caffeic acid related compounds in the standard reaction mixture under UVA irradiation

Fifty percent-inhibition concentrations (IC_{50}) were obtained by measuring concentration dependence of the EPR peak heights of the DMPO/OH radical adduct for caffeic acid related compounds in the standard reaction mixture under UVA irradiation (Table 1). Fifty percent-inhibition concentrations of chlorogenic acid, ferulic acid, salicylic acid, salicylic acid methyl ester, vanillic acid are as follows: chlorogenic acid, 1000 μM; ferulic acid, 1100 μM; salicylic acid, 308 μM; salicylic acid methyl ester, 1100 μM; vanillic acid, 320 μM.

Absorbances of caffeic acid related compounds at 365 nm

To assess the effects of the UVA absorption of caffeic acid related compounds on the inhibition, absorbances at 365 nm were measured for caffeic acid related compounds at IC_{50} (Table 1). Ferulic acid, salicylic acid, salicylic acid methyl ester, vanillic acid hardly absorbed the light at 365 nm. The absorbance of chlorogenic acid was 2.327 at IC_{50}.

Fluorecence spectrum of xanthone (or xanthone with caffeic acid)

To know whether or not caffeic acid quenches the exited xanthone, fluorescence spectra were measured for 15 μM xanthone (or 15 μM xanthone with 10 μM caffeic acid). Xanthone produced emission maxima at λ = 393 nm upon excitation with either 320 nm or 340 nm or 365 nm. The

fluorescence spectrum of xanthone was not affected by the addition of caffeic acid, indicating that caffeic acid does not quench the first excited singlet state of xanthone.

Discussion

The conversion of singlet oxygen ($^1O_2$) to hydroxyl radical ($HO^\bullet$) has been extensively examined during photosensitization of merocyanone 540 (or rose bengal, or anthrapyrazole, or uroporphyrin, or methylene blue) by a spin trapping technique with DMPO [*31–34*]. Addition of catalase resulted in the decrease of the EPR peak height of the DMPO/OH radical adduct in the standard reaction mixture under UVA (365 nm) irradiation [*12*]. The result indicated that H_2O_2 is an intermediate in the formation of DMPO/OH radical adduct in the standard reaction mixture under UVA irradiation [*12*]. On addition of SOD, the EPR peak height of the DMPO/OH radical adduct was enhanced in the standard reaction mixture under UVA (365 nm) irradiation [13], suggesting that the $O_2^{-\bullet}$ is involved in the the formation of DMPO/OH radical adduct. Therefore, the conversion of singlet oxygen ($^1O_2$) to hydroxyl radical ($HO^\bullet$) is not main reaction path in the standard reaction mixture under UVA irradiation.

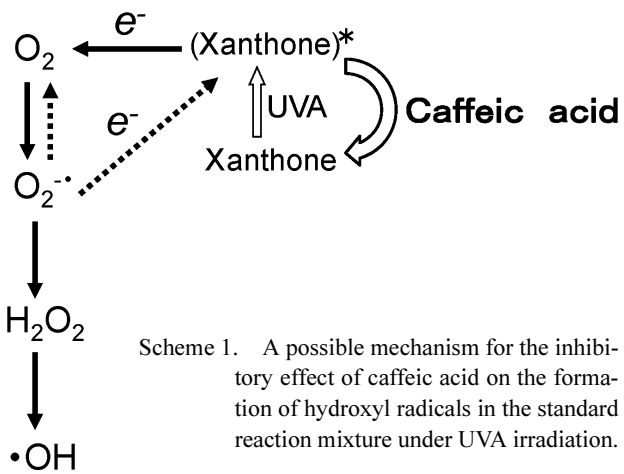
A possible reaction path for the formation of hydroxyl radicals is as follows (Scheme 1) [*12*]. The excited sensitizer (xanthone)*, which forms under UVA irradiation releases an electron to molecular oxygen (Eq. 1). Then, superoxide radical forms. The superoxide radical formed dismutes to form H_2O_2 and molecular oxygen spontaneously (Eq. 2).

$$(\text{Xanthone})^* + O_2 \leftrightarrow (\text{Xanthone})^+ + O_2^{-\bullet} \quad (1)$$

$$O_2^{-\bullet} + O_2^{-\bullet} + 2H^+ \rightarrow H_2O_2 + O_2 \quad (2)$$

The Fenton reaction appears to participate in the standard reaction mixture under UVA irradiation (Eq. 3). The Fe^{2+} in the reaction is possibly supplied through the reduction of Fe^{3+} by superoxide anions [*12*].

$$H_2O_2 + Fe^{2+} \rightarrow HO^\bullet + OH^- + Fe^{3+} \quad (3)$$



Scheme 1. A possible mechanism for the inhibitory effect of caffeic acid on the formation of hydroxyl radicals in the standard reaction mixture under UVA irradiation.

To evaluate at which steps caffeic acid inhibits the formation of hydroxyl radicals in the standard reaction mixture under UVA irradiation, EPR measurements were performed for the standard reaction mixture with EDTA (or SOD) under UVA irradiation (Fig. 2). Caffeic acid could inhibit the $HO^\bullet$ formation (Eq. 3) through chelation of iron ions [*26*, *29*]. Indeed, caffeic acid inhibited the formation of hydroxyl radicals in the Fenton reaction mixture, but not in the presence of EDTA (Fig. 2), suggesting that caffeic acid inhibits the hydroxyl radical formation in the Fenton reaction mixture through chelation of iron ions. On the other hand, on addition of caffeic acid to the standard reaction mixture under UVA irradiation, the EPR peak height of DMPO/OH radical adduct decreased even in the presence of EDTA (Fig. 2). These results indicate that the caffeic acid does not inhibit the $HO^\bullet$ formation through the chelation of iron ions in the standard reaction mixture under UVA irradiation.

To know whether or not caffeic acid scavenges hydroxyl radicals through the hydrogen-donating [*14–24*] or the aromatic hydroxylation [*13*] in the standard reaction mixture under UVA irradiation, caffeic acid was added into the hydroxyl radical generating system, the Fenton reaction mixture with EDTA (Fig. 2F). On addition of 1 mM caffeic acid to the Fenton reaction mixture with EDTA, no EPR peak height change of the DMPO/OH radical adduct was observed. The result indicates that caffeic acid does not scavenge hydroxyl radicals under this reaction conditions. This is due to the high concentration of DMPO (0.1 M) which traps hydroxyl radicals overwhelmingly. Therefore, caffeic acid does not seem to scavenge hydroxyl radicals in the standard reaction mixture under UVA irradiation.

To examine whether or not caffeic acid scavenges the $O_2^{-\bullet}$, EPR measurements were performed for the standard reaction mixture with SOD under UVA irradiation. On addition of caffeic acid to the standard reaction mixture with SOD under UVA irradiation, the EPR peak height of the DMPO/OH radical adduct decreased to $13 \pm 7\%$ of the standard reaction mixture with SOD under UVA irradiation. On the other hand, caffeic acid showed a similar inhibitory effect in the standard reaction mixture without SOD ($6 \pm 2\%$) under UVA irradiation. In the presence of SOD, the reaction (Eq. 2) is fast enough to ignore the effect of caffeic acid. If caffeic acid scavenges the $O_2^{-\bullet}$ (Eq. 2) in the absence of SOD, the scavenging effect could be reduced in the presence of SOD. This is not the case for this reaction. Therefore, caffeic acid does not appear to scavenge the $O_2^{-\bullet}$ in the standard reaction mixture under UVA irradiation.

To assess whether the inhibitory effect is due to the UVA

absorption of caffeic acid, and caffeic acid related compounds or not, UVA (365 nm) absorbances of these compounds were measured at IC_{50}. Caffeic acid, ferulic acid, salicylic acid, salicylic acid methyl ester and vanillic acid hardly absorbed light at 365 nm, suggesting that the inhibitory effect is not due to the absorption of these compounds at 365 nm (Table 1). Therefore, caffeic acid hardly inhibits the step from xanthone to (xanthone)*.

Caffeic acid has no influence on all the steps in the Scheme 1 except for the step from (xanthone)* to xanthone. Thus, caffeic acid seems to inhibit the formation of hydroxyl radicals in the standard reaction mixture under UVA irradiation through a novel antioxidant action, *i.e.*, ability to quench the excited xanthone. Fluorescence spectrum of xanthone (or xanthone with caffeic acid) indicated that caffeic acid does not quench the first excited singlet state of xanthone. Caffeic acid may quench the excited triplet state of xanthone which can interact with the triplet state of oxygen to form superoxide. Since the excited triplet state of xanthone is generally stable compared with singlet one, It can be formed from the excited singlet state of xanthone.

Of the compounds examined, caffeic acid, chlorogenic acid, ferulic acid, salicylic acid, salicylic acid methyl ester and vanillic acid showed the inhibitory effect. Since these compounds have phenol moieties, the phenol moieties may be essential for the inhibitory effects. Of the compounds examined, caffeic acid is the most potent inhibitor. That may be due to the intermolecular interaction between caffeic acid and xanthone.

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